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NUCLEOTIDE SEQUENCES SPECIFIC TO YERSINIA PESTIS AND METHODS FOR THE DETECTION OF YERSINIA PESTIS

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[0001] The United States Government has rights in this invention pursuant to Contract No. W-7405-ENG-48 between the United States Department of Energy and the University of California for the operation of Lawrence Livermore National Laboratory.

RELATED APPLICATION

[0002] This application claims the benefit of U.S. Provisional Application No. 60/400,890 filed August 1, 2002, and entitled, "DNA Diagnostics Yersinia Pestis and Methods for the Detection of Yersinia Pestis" which is incorporated herein by reference.

GENE SEQUENCE LISTING IN COMPUTER READABLE FORM

[0003] The sequence listing information recorded in computer readable form is identical to the written on paper sequence listing.

BACKGROUND

[0004] *Yersinia Pestis* is the species of bacteria known to cause the plague, a highly contagious and often fatal disease. Since the attack on the World Trade Center in New York of September 11, 2001, there has been a growing concern that terrorists or rogue governments will use the *Yersinia pestis b*acterium as a weapon of mass destruction and instrument of terror. Since the events of September 11, 2001, the United States Government has been developing

reliable methods and systems to detect the *Yersinia pestis* bacterium so that immediate and effective counter measures can be undertaken. The existing methods for detecting the *Yersinia pestis* bacterium are considered inadequate because of the higher than acceptable rate of false positive and false negative results. False positive results lead to confusion regarding whether the *Yersinia pestis* bacterium is actually present and whether protective measures should immediately be implemented. Conversely, false negative results would allow the *Yersinia pestis* bacterium to remain undetected with consequent adverse impacts. A more reliable method of detecting the *Yersinia pestis* bacterium would reduce the occurrence of false positive and false negative results and provide decision makers with greater confidence in implementing appropriate counter measures.

SUMMARY OF THE INVENTION

[0005] This invention includes the nucleotide sequences that are identified in SEQ ID NOs 4, 8, 12, 16, 20 and 24 that are specific to *Yersinia pestis.*

[0006] Another aspect of the invention includes a Forward Primer, the nucleotide sequences that are identified in SEQ ID Nos 1, 5, 9, 13, 17 and 21, and any primers that are derived from these nucleotide sequences.

[0007] A further aspect of the invention includes a Reverse Primer, the nucleotide sequences that are identified in SEQ ID NOs 2, 6, 10, 14, 18 and 22, and any primers that are derived from these nucleotide sequences.

[0008] A further aspect of the invention includes a Hybridization Probe, the nucleotide sequences that are identified in SEQ ID NOs 3, 7, 11, 15, 19 and 23, and any probes that are derived from these nucleotide sequences.

[0009] This invention also includes a method for the detection of *Yersinia pestis* using the bacterium specific nucleotide sequence comprising: providing a sample in an environment that is suitable for isolating genomic DNA for amplification using PCR and under conditions suitable for hybridization with a least one group of nucleotides consisting of a forward primer, a reverse primer and a hybridization probe and detecting the existence of *Yersinia pestis* specific nucleotide sequences by a nucleotide detection method, such as PCR and flurogenic 5' nuclease PCR assay, wherein the existence of the nucleotide sequence indicates the presence of *Yersinia pestis* in the sample.

BRIEF DESCRIPTION OF THE SEQUENCE SETS

SEQ ID NO:1	Primer
SEQ ID NO:2	Primer
SEQ ID NO:3	Probe
SEQ ID NO:4	Amplicon
SEQ ID NO:5	Primer
SEQ ID NO:6	Primer
SEQ ID NO:7	Probe
SEQ ID NO:8	Amplicon
SEQ ID NO:9	Primer
SEQ ID NO:10	Primer
SEQ ID NO:11	Probe
SEQ ID NO:12	Amplicon
SEQ ID NO:13	Primer

SEQ ID NO:14	Primer
SEQ ID NO:15	Probe
SEQ ID NO:16	Amplicon
SEQ ID NO:17	Primer
SEQ ID NO:18	Primer
SEQ ID NO:19	Probe
SEQ ID NO:20	Amplicon
SEQ ID NO:21	Primer
SEQ ID NO:22	Primer
SEQ ID NO:23	Probe
SEQ ID NO:24	Amplicon

DETAILED DESCRIPTION

[00010] Disclosed herein are six nucleotide sequences located on different loci of the *Yersinia pestis* bacterium genome. Also disclosed, are primers and the hybridization probes used in detecting the specific nucleotide sequences as well as method for identifying *Yersinia pestis* by analyzing samples taken from monitoring devices, such as air monitors, for the nucleotide sequences that are specific to *Yersinia pestis.* By using the primers and hybridization probes developed from the nucleotide sequences identifies as unique to the Yersinia Pestis bacterium to detect the presence of the *Yersinia pestis* bacterium, far more reliable results are obtained than by using existing methods. False positive and false negative results are also greatly reduced.

[00011] *Yersinia Pestis* is the bacterium that causes what is commonly known as the plague, a disease that is contagious and can be fatal if not detected early and treated with appropriate antibiotics. The symptoms of plague "are fever, headache, weakness, and rapidly developing pneumonia with shortness of breath, chest pain, cough, and sometimes bloody or watery sputum. The pneumonia progresses for 2 to 4 days and may cause respiratory failure and shock." This information can be found at the internet address:www.bt.cdc.gov/documentsapp/FactSheet/Plague/About.asp. It is on the Center for Disease Control and Prevention (CDC) list of possible bacteria that has potential as a biological warfare weapon. The CDC has developed a list of possible pathogens that may be used as bioterrorism weapons. *Yersinia Pestis* has been listed in Category A of possible diseases and agents. Those diseases and agents in Category A are considered a high risk to national security because they "can be easily disseminated or transmitted from person to person; result in high mortality rates and have the potential for major public health impact; might cause public panic and social disruption; and require special action for public health preparedness." This information can be found at the internet address: www.bt.cdc.gov/agent/agentlist-category.asp.

[00012] A key element in developing defenses against the use of *Yersinia pestis* is the ability to quickly and accurately detect the presence of the bacterium. Early detection will allow for the implementation of effective counter measures. Additionally, early detection will allow for the identification and treatment of those that may have been exposed to the bacterium. Early detection and treatment is essential for the treatment of Plague because significant adverse health effects, including death, may occur if it is not detected early and treated with antibiotics.

[00013] Existing detection methods have resulted in a higher than acceptable rate of false positive and false negative results. Such results are inadequate and can create confusion

regarding the appropriate countermeasures, if any, that should be undertaken because it is unclear whether the bacterium is present or not. If the bacterium is not present, undertaking counter measures may cause undue expense and create unwarranted concern among those that may incorrectly believe they have been exposed.

[00014] Although the genome for *Yersinia pestis* has already been mapped, this alone was not sufficient to develop a reliable and accurate detection mechanism because the current methods use nucleotide sequences that may be common to many different bacteria. Thus, existing detection methods could not distinguish between various bacteria, which resulted in higher than acceptable false positive detection rates. Similarly, some existing detection methods resulted in false negative results because they were not sensitive enough to detect the bacterium. Using a nucleotide sequence that is specific to the *Yersinia pestis* bacterium results in a move reliable detection method.

[00015] Six nucleotide sequences contained in SEQ ID Nos 4, 8, 12, 16, 20 and 24 that are specific to the *Yersinia pestis*. These sequences are known as "amplicons." The existence of these nucleotide sequences in a sample is proof that the bacterium *Yersinia pestis is* present. In order to detect any of the six amplicons specific to *Yersinia pestis,* a series of forward and reverse primers and hybridization probes were developed for each of the six amplicons.

[00016] The typical assay determines the presence of SEQ ID Nos 4 and 8 using the sequence specific primers and the hybridization probes. If there is a positive result for the presence of *Yersinia pestis* then an assay is run to determine the presence of additional amplicon sequences, as a means to double check for the presence of *Yersinia pestis.*

[00017] Once the *Yersinia pestis* specific nucleotide sequences were identified, the presence of the bacteria could be detected from environmental samples using PCR assay analysis and

detection. PCR is a technique utilized to amplify genomic DNA. Typical PCR reactions include appropriate PCR buffers, nuclease polymerase and one or more oligonucleotide primers and hybridization probes. Various modifications of PCR techniques are possible as detailed in Current Protocols in Molecular Biology ed. F.M. Ausubel, R. Brent, D.D. Moore, K. Struhle, Massachusetts General Hospital and Harvard Medical School (1987), which is hereby incorporated by reference. The following US patents describe PCR and are incorporated herein by reference: US 4,683,195; US 4,683,202; US 4,800,159.

[00018] One method that may be used for real-time PCR amplification and detection is TaqMan®. The principles involved in the conventional Taqman® 5' exonuclease assay are described in detail by Holland et al in, *Detection of specific polymerase chain reaction product by utilizing the 5'----3' exonuclease activity of Thermus aquaticus polynucleotide polymerase*, Proc Natl Acad Sci U S A 88 (16):7276-80, 1991, which is herein incorporated by reference. TaqMan® real time detection can also be used to simultaneously detect a plurality of nucleic acid targets when it is used with multiplex PCR, which enables simultaneous detection of more than one target sequence, thus enhancing detection accuracy. A few examples of typical PCR instruments include the ABI prism 7700, the Cepheid Smart Cycler, and the Bio-Rad iCycler.

In order to use a PCR assay method for detection of the *Yersinia pestis* bacterium, the sample must be prepared to extract all DNA that may be present. The following is a protocol for the preparation of samples taken from ambient air monitoring devices for nucleotide detection using fluorogenic 5' nuclease PCR assay.

Assay Protocol

Definitions:

DNA-deoxyribonucleic acid

EDTA-ethylenediaminetetraacetic acid

PCR-polymerase chain reaction

PCR water-autoclaved water, then filtered

CT-cycle threshold-the cycle in which the fluorescence signal crosses a user defined threshold

FAM-reporter dye

TAMRA-quencher dye

Sample Preparation:

[00019] Exposed environmental filter are suspended in Sodium phosphate/EDTA, Tween buffer and bead beaten. The supernatant is filtered and washed to yield the genomic DNA extract. The extract is then subjected to real-time polymerase chain reaction (PCR) assay using a fluorescent-labeled probe. This process monitors a PCR reaction and the quantity of double-stranded product that is produced

Materials Needed:

1. A series of forward and reverse primers, a hybridization probe and polymerase reagents specific to the first amplicon to be detected.
2. A series of forward and reverse primers, a hybridization probe and polymerase reagents specific to the second amplicon to be detected.

Bead beater Kit includes:

a. 3 capped tubes containing a filter and beads
b. 3 yellow ultra free MC Centrifugal Filter Units
c. 6 blue microcon YM-100 filter units

d. 12 collection tubes

e. 7 PCR reaction mix-includes primer/ probe and Taq-labeled A,B,C,D,E,F,G

f. 48 –25 μl Smart Cycle reaction tubes

g. Sodium phosphate buffer/EDTA Teen buffer

h. PCR water

i. Inhibitory control DNA

j. Extra unlabeled tubes

3. Cepheid Smart Cycle

4. Microcentrifuge

5. Microfuge for Cepheid tubes

Preparation of DNA Extract:

This part of the assay protocol should be perform in segregated work areas and in a biosafety cabinet using BSL 2 practices.

1. Add 400 μl of Sodium phosphate/EDTA Teen buffer to each of the capped tubes containing a filter and beads. Screw cap tightly.

2. Insert tubes one at a time into the bead beater.

3. Bead beat the capped tube for 3 minutes and a speed of 5000 rpm.

4. Remove capped tube from bead beater and place the tube on ice for a minimum of 2 minutes to cool.

5. Wash Steps.

- Spin capped filter tubes for 10 seconds (pulse spin) in microcentrifuge.

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- Transfer approximately 400 μl of the supernatant to the yellow top filter collection tube.
- Spin the yellow top filter tube with the supernatant @ 7000 rpm for 3 minutes.
- Transfer the filtered liquid to a blue microcon filter on collection tube #1.
- Spin @ 7000 rpm for 1 minute. Check fluid level in the blue microcon filter. If it is above the white base, pulse spin for about 10 seconds to bring the level at or a little below the white area. Several pulse spinsmay be necessary to bring the level down. This is approximately 100-200 μl of liquid.
- Transfer this liquid on the top of the filter to a second blue microcon filter on a clean collection tube #2. Tilt the tube at a 45° angle and take off the liquid-do NOT vacuum the filter.
- Add 400 μl of PCR water to the second blue microcon filter with the added liquid.
- Spin @ 7000 rpm for 2 minutes. Do not be spin dryApproximately 50-100 μl of liquid should be on top of the filter. Pulse spin if the level is too high
- Using clean, metal forceps remove the blue microcon filter from the collection tube #2 and place the blue microcon filter on a clean collection tube #3. Discard collection tube #2.
- Add 400 μl PCR water to the blue microcon filter on collection tube #3.
- Spin @ 7000 rpm for 2 minutes. Do not be spin dry; approximately 50-100 μl of liquid should be on top of the filter. Pulse spin if the level is too high.
- Remove the blue microcon filter. Place the blue microcon filter on collection tube #4. Discard collection tube #3

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- Add 400 μl PCR water to the blue microcon filter on collection tube #4.
- Spin for 1 minute @ 7000 rpm. Check fluid level in the blue microcon filter. If it is above the white base, pulse spin for about 10 seconds to bring the level at or a little below the white area. Several pulse spins may be necessary. If the level of liquid is at or a little below the white base, there is approximately 100-200 μl of DNA extract.

If for some reason the pulse spin has brought the level of DNA extract down too low add 200 μl PCR water and bring the level carefully to the white base level by pulse spinning for less time.

- Transfer the liquid on the top of the blue microcon filter (i.e., the DNA extract) to the eppendorf tube.
- If the PCR assay cannot be performed immediately, keep extract refrigerated.

PCR Assay:

1. Thaw on ice each set of primer/probe/Taq polymerase sets. Once thawed PCR assay must begin. Do NOT refreeze. Keep on ice while testing.

2. Add 20 μl of each of the PCR reaction mixes for each amplicon and one inhibitory control to the appropriately labeled Cepheid reaction tubes; e.g. 1-1A is for amplicon 1, filter 1, a set of primers, probe, Taq polymerase.

    a. Add 5 μl of DNA extract to each of the tubes-rinse tip 1-2 times in the mix and discard the tip.
    b. Use a clean tip for each reaction tube.
    c. Each tube should have a total of 25 μl .

3. Add 15 μl of PCR reaction inhibitory mix to appropriately labeled Cepheid tubes; e.g, INHIB.

a. Add 5 μl DNA extract to each tube-rinse tip 1-2 times in the mix and discard tip.

a. Add 5 μl of DNA inhibitory control to each tube-rinse tip 1-2 times in the mix and discard the tip.

a. Use a clean tip for each reaction tube.

b. Each tube should have a total of 25 μl.

4. Include as controls:

NTC (no template control) for each set of primers/probe, Taq.

5. Spin Cepheid tubes in Cepheid microfuge for about 4 seconds. This mixes the PCR reaction mix and DNA into the optic diamond area. Check to see that the optic area is filled.

6. Run Cepheid Smart Cycler

7. Record all CT values (including 0) on the result sheet for the appropriate organism and filter. CT values equal to 34 to 35 indicate negative readings-no *Yersinia pestis* detected. CT values below 34 indicate a positive reading-*Yersinia pestis* DNA detected.

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yersinia pestis Table 1
environmental soil samples

	Y. pestis-kim DNA/Positive Control	Environmental Soil Sample D4		D36		D65	
		run1	run2	run1	run2	run1	run2
Seq.ID No.1,2,3	23.28	35	35	35	35	35	35
Seq.ID No 5,6,7	20.28	35	35	35	35	35	35
Seq.ID No.9,10,11	19.78	35	35	34.43	35	35	35
Seq.ID No.13,14,15	20.17	35	35	35	35	35	35
Seq.ID No.17,18,19	32.57	35	35	35	35	35	35
Seq.ID No.21,22,23	22.26	35	35	35	35	35	35

	Y. pestis-kim DNA/Positive Control	Environmental Soil Sample D411		D425		D428	
		run1	run2	run1	run2	run1	run2
Seq.ID No.1,2,3	23.28	35	35	35	35	35	35
Seq.ID No 5,6,7	20.28	35	35	35	35	35	35
Seq.ID No.9,10,11	19.78	35	35	35	35	35	35
Seq.ID No.13,14,15	20.17	35	35	35	35	35	35
Seq.ID No.17,18,19	32.57	35	35	35	35	35	35
Seq.ID No.21,22,23	22.26	35	35	35	35	35	35

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[00020] Table 1 shows the results of assay runs that were performed using the above described protocol. An assay set containing the primers and probe for each amplicon sequence was added to a sample containing either the Yersinia pestis DNA or an environmental soil sample. The environmental soil samples were used as controls to demonstrate that the primers and probes derived from the Yersinia pestis specific amplicon could identify the Yersinia pestis bacterium, thereby demonstrating the specificity of the amplicon sequence. The Yersinia pestis DNA was obtained from American Type Culture Collection. The assay sets containing the probes and primers were obtained from various vendors such as ABI and Biosearch.

[00021] Some assays were run twice for a specific environmental soil sample, while some assays were only run once. The results show that the assay runs having the Yersinia pestis DNA in the sample had positive results, CT values of less than 34. Those assay runs with environmental soil samples had negative results, CT values of 34 to 35, an expected result given that Yersinia pestis bacterium would not be expected to be present in a soil sample. These results show that the primers and probes derived from the Yersinia pestis specific amplicon can distinguish between DNA from the Yersinia pestis bacterium and DNA that may be contained in environmental soil samples, thereby, demonstrating that the nucleotide sequences in the amplicons are specific to Yersinia pestis and that false positive results should not occur using these assays in the field.

[00022] The inventions disclosed herein are based on nucleotide sequences that are specific to *Yersinia pestis*. Accordingly, although air monitors are an effective method of obtaining samples for analyses, a wide variety of other media and methods may be used to

provide the samples for analysis for the *Yersinia pestis* bacterium and this invention is not limited by the method or media from which a sample for analysis is obtained.

[00023] All numbers expressing quantities of ingredients, constituents, reaction conditions, and so forth used in the specification and claims are to be understood as being modified in all instances by the term "about". Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the subject matter presented herein are approximations, the numerical values set forth in the specific examples are reported as precisely as possible. Any numerical value, however, inherently contain certain errors necessarily resulting from the standard deviation found in their respective testing measurements.

[00024] All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.